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The overall objective of this project is to develop an assay to detect small numbers of breast cancer cells in a patient's peripheral blood. This assay would be used to test the hypothesis that the presence of circulating breast cancer cells would be predictive of stage at presentation or relapse. The first stage of this project has been to optimize a quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) to quantify breast cancer-specific RNA molecules in the peripheral blood. The result of this initial work was to select keratin 19 (K19) RNA as the best target for detecting small numbers of breast cancer cells. We have also selected an internal reference RNA (β_2-microglobulin), and we have optimized our protocol for extracting RNA from patient blood samples. The second phase of this project has been to develop methods to enrich blood samples for tumor cells, thereby increasing the sensitivity of the assay. Having optimized these parameters, in the third phase we are using the qRT-PCR assay for K19 RNA to study patients with breast cancer, both at the time of presentation and after treatment.

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INTRODUCTION

The overall objective of this project was to develop an assay to detect small numbers of breast cancer cells in a patient's peripheral blood. This assay was used to test the hypothesis that the presence of circulating breast cancer cells is predictive of stage at presentation or relapse. The first stage of this project was to optimize a quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) to quantify breast cancer-specific RNA molecules in the peripheral blood. The result of this initial work was to select keratin 19 (K19) RNA as the best target for detecting small numbers of breast cancer cells. We also selected an internal reference RNA (β_2-microglobulin), and we optimized our protocol for extracting RNA from patient blood samples. The second stage of this work was to develop ways to enrich peripheral blood specimens for tumor cells, thereby increasing the sensitivity of the assay. Having optimized these parameters, in the third phase we are using the qRT-PCR assay for K19 RNA to study patients with breast cancer, both at the time of presentation and after treatment.

WORK ACCOMPLISHED

Task 1: To develop molecular tests to quantify breast cancer cells in the peripheral blood

In the first year of the project, we devoted our efforts to optimizing the qRT-PCR assay to detect small numbers of circulating breast cancer cells. For this task, we designed PCR primers and probes, identified a cell line containing the RNA targets, optimized PCR conditions, evaluated the PCR method for its ability to quantify the target RNA, selected an internal reference RNA, optimized methodology for purifying RNA from peripheral blood, and used the optimized assay to quantify the RNA target in normal peripheral blood to establish a reference range. In the second year of the project, we devoted our efforts to assay optimization. Specifically, we developed methodology for increasing assay sensitivity by enriching for circulating epithelial cells. In the final year of the project, we performed some additional optimization, including adjusting components of the reaction buffer to increase the signal-to-noise ratio and adding a step to the RNA isolation procedure to eliminate DNA contamination, which was a possible source of false-positive results.

Year 1: Assay Development

Primer and probe design

Taqman primers and probes were designed to detect K19 and MUC1 using PrimerExpress 1.0 software (Applied Biosystems). K19 was selected since it is expressed in neoplastic epithelial cells (including breast cancer cells) and not in blood cells. MUC1 was selected since it is known to be expressed in breast cancer, and since the MUC1 protein is used as a serologic tumor marker to determine patient tumor burden. Primers and probes were also designed for three RNAs that were to be tested as internal reference RNAs. The prospective reference RNAs were ribosomal RNA (rRNA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β2-microglobulin (β2m). All primers and probes were designed to span an intron, which would prevent unwanted detection of DNA. In addition, since it is known that there are a number of pseudogenes for K19 (1, 2), K19 primers were designed to detect only the "genuine" K19 RNA and not the pseudogenes. The primers and probes are listed in Table 1.

Identification of a cell line expressing breast cancer RNAs

Since a significant amount of breast cancer RNA would be required to optimize the qRT-PCR assay, we needed a source of breast cancer RNA that could be maintained in the laboratory. Thus, we evaluated two breast cancer cell lines for the presence of K19 and MUC1 RNA. The cell lines used were SKBR3 and BT20. Both cell lines were found to contain ample K19 and MUC1 RNA, so the SKBR3 line was selected for further study since it was the easiest to maintain in tissue culture.

Optimization of qRT-PCR

For each target RNA, a series of PCR reactions was performed in which the concentrations of the forward and reverse primers were varied from 100 nM to 900 nM. The probe concentration was held constant at 100 nM. The maximum amount of PCR product formed for the targets tested was obtained when the primer concentrations were 900 nM for both the forward and reverse primers. By using these primer concentrations and varying the probe concentration, we determined that the optimum probe concentration was 100 nM for all primer/probe sets.

Selection of RNA target for qRT-PCR

We initially tried K19 and MUC1 as targets for quantification using RNA isolated from SKBR3 and BT20 cells. We found that K19 was approximately 100-fold more sensitive a marker for breast cancer than MUC1, most likely due to its higher abundance in breast cancer cells.

Having established the PCR conditions, we performed qRT-PCR on RNA isolated from BT20 cells. Serial 10-fold dilutions of total RNA were prepared, and qRT-PCR was performed using 10 ng down to 10 fg of total RNA. The results of this experiment are shown in Figure 1. Figure 1A shows amplification curves using K19 primers and probes. As the PCR products accumulate, the fluorogenic probe anneals to the PCR products. Taq polymerase, due to its 5'→3' exonuclease activity (3), digests probe that is annealed to the PCR products. The probe contains a fluorochrome (FAM) at the 5' end and a fluorescent quencher (TAMRA) on the 3' end. The intact probe generates very little fluorescent signal, but when the probe is digested by Taq polymerase, FAM is spatially separated from TAMRA, resulting in an increase in the fluorescent signal. Thus as the PCR products accumulate, fluorescence increases as shown in Figure 1A. The cycle at which the fluorescence signal increases above a given threshold (the horizontal line in Figure 1A) is inversely proportional to the starting amount of RNA. This cycle is referred to as the "threshold cycle", or Ct. Figure 1B shows that there is a linear relationship between Ct and the log of the starting RNA quantity. The assay is linear from 10 ng down to 1 pg of total RNA. 1 pg of RNA corresponds to about 1 breast cancer cell. When a similar experiment was performed using MUC1 as a target, the assay could only detect a MUC1 signal in 100 pg of RNA. Thus the sensitivity of the MUC1 assay is 2 logs lower than that of the K19 assay. Therefore, we have focused our efforts on optimizing the K19 assay for use in this study.

The above experiment was performed by diluting breast cancer cell line RNA in water. However, in actual use, breast cancer RNA will be mixed with RNA from other blood cells. Therefore, we repeated the serial dilution experiment, this time mixing SKBR3 RNA with RNA from a hematopoietic cell line, K562. A constant amount of RNA (50 ng) was used in each assay. Under these conditions, we were able to detect the equivalent of 2.5 ng of SKBR3 RNA, which we consider the limit of detection for this assay.

Table 1. Taqman primers and probes

RNA Target	Forward Primer	Reverse Primer	Probe
K19	GCGGCGCACC CTTCA	GTTTCTGCCA GTGTGTCTTC CA	CAGTCACAGCTGAGCATGAA AGCTGCC
MUC1	TGCGCTGGCC ATTGTCT	AGCTGCCCGT AGTTCTTTCG	TCTCATTGCCTTGGCTGTCT GTCAGTG
rRNA	CGGCTACCAC ATCCAAGGAA	GCTGGAATTA CCGCGGCT	TGCTGGCACCAGACTTGCCC TC
GAPDH	CCACATCGCT CAGACACCAT	CCAGGCGCCC AATACG	AAGGTGAAGGTCGGAGTCA ACGGATTTG
β2-microglobulin	TGCTCGCGCT ACTCTCTCTTT	GGATGACGTG AGTAAACCTG AATCT	CCTGGAGGGCATCCAGCGT ACTCC

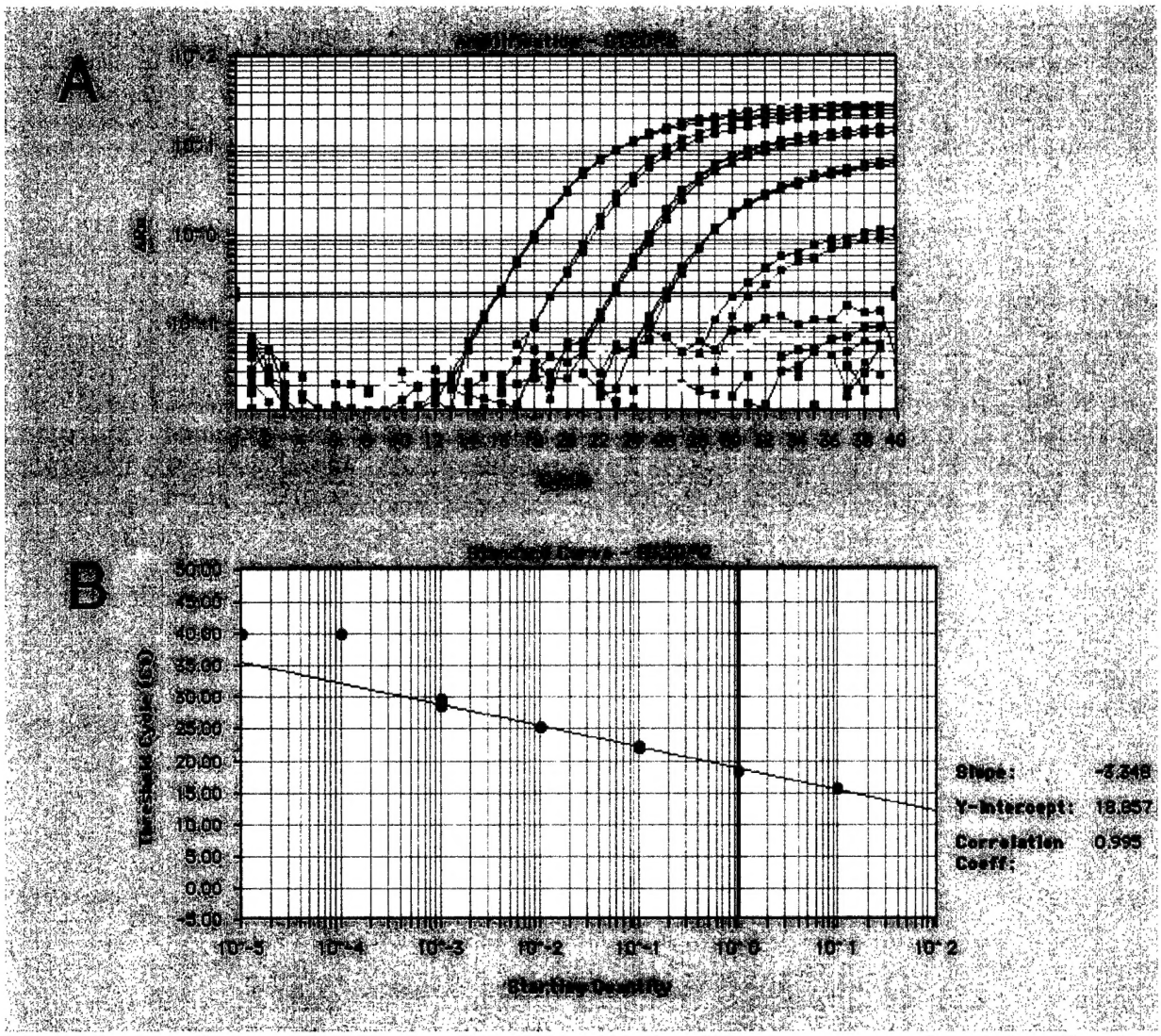

Figure 1. Quantitative RT-PCR for K19 RNA. (A) Amplification curves of serially diluted RNA. RNA isolated from BT20 breast carcinoma cells was added in triplicate to the K19 qRT-PCR assay. Serial 10-fold dilutions were used starting with 10 ng and ending with 10 fg. The curves represent FAM fluorescence intensity relative to an internal reference dye (ROX). The PCR cycle at which the fluorescence intensity becomes greater than the background threshold (horizontal black line at 0.219 fluorescence units is measured as the "Ct". (B) Standard curve generated with the data shown in panel A. Note that the relationship between the log of RNA quantity vs. Ct is linear from 10 ng down to 1 pg of RNA with an excellent correlation coefficient (0.995).

Selection of an internal reference RNA

An internal reference RNA is used as a normalization control to correct for variations in RNA loading and other factors that might affect PCR efficiency for a given RNA sample. An ideal reference RNA is one that is present in all cells at constant levels, is comparable in quantity to the test RNA (i.e., K19), and for which primers and probes can be designed easily. We evaluated rRNA, GAPDH, and β2m RNAs as possible internal reference RNAs. rRNA was present at too high a level for accurate quantification. GAPDH is also expressed at a high level. In addition, there are multiple pseudogenes for GAPDH in the genome (4, 5), making it difficult to design primers and probes. Finally, β2m has been used successfully as an internal reference RNA for a RT-PCR

for BCR-ABL currently in use in our laboratory (6), so we have experience with this gene. When used as a target for qRT-PCR, β2m gave reproducible amplification. For these reasons, we have selected β2m as our internal reference RNA.

We investigated whether we could simultaneously amplify K19 and β2m in a single multiplex PCR reaction, but the sensitivity to detect K19 RNA decreased by 100-fold, so we decided to perform the two PCR reactions separately.

Optimization of RNA preparation

To test the clinical sensitivity of the qRT-PCR assay for K19 RNA, we added known numbers of SKBR3 cells to normal peripheral blood samples. The blood samples were made to contain from 1 in 100 to 1 in 10^6 SKBR3 cells per white blood cell. RNA was prepared from the spiked specimens and subjected to qRT-PCR.

RNA was initially prepared from whole blood, but this resulted in the ability to detect only 1 SKBR3 cell in 100 white cells. This level of sensitivity is unacceptably low for detecting minimal numbers of breast cancer cells in the peripheral blood. We investigated whether the RNA preparation method would affect sensitivity. Purifying RNA using a phenol-based method (Trizol) or using RNA preparation kits from Qiagen and Gentra Systems resulted in similarly low sensitivity. Furthermore, RNA prepared using the Gentra Systems kit resulted in significant levels of DNA contamination. Some DNA contamination was observed with the Qiagen kit as well. To determine if DNA contamination affected assay sensitivity, a DNase step was added. This resulted in less DNA contamination, but this step did not improve the sensitivity of the assay, so it was not used in subsequent experiments at this time. However, after additional experience, a DNase step was reintroduced later (see below).

We considered the possibility that red blood cell contaminants might be adversely affecting the sensitivity of the K19 RT-PCR assay, so we added a Ficoll gradient density centrifugation step to remove red cells and consequently enrich for white cells (and presumably cancer cells as well). We tried two Ficoll methods, 1) Vacutainer tubes (Becton-Dickinson) containing a Ficoll preparation and a solid-phase cell separator layer, and 2) standard Ficoll gradients made in the laboratory. The Vacutainer tubes were found to produce false-positive results, i.e., K19 signals were obtained from normal peripheral blood that had not been spiked with tumor cells. In contrast, using Ficoll gradients made in the laboratory, we were able to get cleanly negative unspiked controls, and we were able to detect as few as 5 SKBR3 cells in 10^6 white cells. This level of sensitivity approaches the theoretical limit of PCR sensitivity, and thus will be acceptable for a clinical assay.

Effect of order in which blood tubes are drawn

We performed a pilot study to determine whether we needed to be concerned about contamination of blood samples with epidermal cells. It is theoretically possible that the presence of skin cells could yield false positive results. To make this determination we collected two tubes of blood from 10 normal subjects and performed qRT-PCR for K19 RNA on both tubes. K19 mRNA was present at very low levels in some normal subjects, but that level was not significantly higher in the first tube, as might be expected if contamination with skin cells was a significant analytical problem.

Year 2: Assay Improvement

During the second year, we devoted our efforts to increasing the sensitivity of the qRT-PCR assay to detect small numbers of circulating breast cancer cells. In previous section, I indicated that we were able to detect as few as 5 tumor cells in 10^6 white cells. This level of sensitivity was based on experiments in which tumor cells grown in culture were mixed with non-tumor cells. The sensitivity of the assay was higher when tumor cell RNA was mixed with water, indicating that the presence of background RNA decreases the sensitivity of the RT-PCR reaction. Thus, I determined that if we could reduce the background RNA in the assay, we should improve the assay's sensitivity. This was the rationale for our efforts of the second year, in which we attempted to enrich samples for tumor cells prior to RNA isolation.

Ficoll-Hypaque Density Gradient Separation

Ficoll-Hypaque can be used to separate whole blood based on cell density. Red blood cells and granulocytes are denser than standard Ficoll-Hypaque, so these cells can be removed by layering whole blood over a Ficoll-Hypaque cushion, and then performing centrifugation. Our initial experiments using whole blood spiked with SKBR3 cells showed that RNA isolated from mononuclear cells that "float" on Ficoll-Hypaque was amplified by PCR more efficiently than RNA isolated from spiked whole blood. Additional efficiency was obtained when red cells were lysed with hypotonic ammonium chloride prior to Ficoll-Hypaque separation. The increase in assay sensitivity provided by Ficoll separation was nearly 4 logs, to 5 tumor cells in 10^6 white cells.

Positive Selection with Magnetic Beads

Magnetic bead enrichment had been used successfully to selectively purify epithelial cells from blood (7). To test the utility of magnetic beads to enrich for breast cancer cells in blood we used Dynabeads (Dynal Biotech, Oslo, Norway) to purify SKBR3 cells that had been added to whole blood samples. Successful enrichment was verified using flow cytometry. In an experiment in which qRT-PCR was performed in parallel on Ficoll-enriched and Dynabead-enriched samples, the Dynabeads produced a 10-fold increase in sensitivity, although the sensitivity of this experiment was lower than in most experiments. The enrichment for epithelial cells was verified by flow cytometry. Despite the enrichment observed, there was still considerable contamination of the enriched cells by leukocytes as evident from the β_2-microglobulin signal obtained from the enriched specimens, from the flow cytometry results that show significant numbers of non-epithelial cells, and from microscopic examination of the enriched cell specimens.

Negative Selection with RosetteSep

Because of the leukocyte contamination observed with Dynabead selection, and because it was possible that the Dynabeads were less than 100% efficient at recovering tumor cells, we explored the possibility of depleting leukocytes rather than enriching for tumor cells. RosetteSep is a product of Stem Cell Technologies (Vancouver, BC, Canada) that uses a mixture of anti-leukocyte antibodies to remove white cells by rosetting followed by density gradient separation. We compared the sensitivity of the magnetic bead enrichment and RosetteSep negative selection methods on blood samples spiked with SKBR3 cells. While magnetic bead enrichment appeared to result in higher amounts of K19 mRNA in samples spiked with as few as one SKBR3 cell per million white cells, the RosetteSep method gave a detectable K19 signal with one SKBR3 cell per ten million white cells. We need to explore further which method is optimal for maximizing clinical sensitivity of the K19 qRT-PCR assay.

Establishment of reference range

Having optimized specimen processing parameters, we sought to establish a reference range for K19 mRNA with normal blood donors. Preliminary experiments using either positive or negative selection enrichment methods indicated that K19 mRNA was present at very low levels in the peripheral blood of normal volunteers. Therefore, it was important to establish the maximum amount of K19 mRNA that is present in normal individuals to determine the significance of finding a given level of K19 mRNA in breast cancer patients. The result of this study was that 95% of normal subjects had less than 2.38 ng of SKBR3 RNA equivalents per 10 ml of blood.

Year 3: Further method refinement

The K19 qRT-PCR was in its final form after the second year, however there were two issues that we continued to work on in the final year of the project. The first was the presence of a low K19 background signal in some of our controls that had no reverse transcriptase added (the no-RT controls). This background suggested that the presence of small amounts of contaminating DNA was contributing to the K19 signal. Most likely this was due to the presence of processed K19 pseudogenes (1, 2). To address this problem, we tried treating our RNA preparations with DNase. As a result, the low background signal we observed in our no-RT controls disappeared with no loss of signal from the K19 RNA present.

The other area we made progress on in the final year was to optimize the reaction master mix used for RT-PCR. For the ABI 7700, the fluorescence signal is normalized to an internal reference dye, ROX, which is coupled to a non-reactive oligohexamer included in each reaction. It turned out that we were using too little of the internal reference dye, which resulted in increased background signals in our assay. Between the use of DNase and increased amounts of internal reference dye, we have significantly improved the signal-to-noise ratio of the K19 qRT-PCR assay.

Task 2: To apply the test to untreated breast cancer patients

As of 10 October 2002, we have performed K19 qRT-PCR on 76 patients whose blood samples were processed to enrich for epithelial cells by one of the two methods. We have collected specimens on an additional 26 patients; these results are pending. Complete data analysis has been performed based on the results of 58 breast cancer patients and 62 normal controls. 38 breast cancer patients (66%) and 34 controls (55%) had detectable K19 mRNA in their peripheral blood (> 2.5 ng SKBR3 cell equivalents per 10 ml blood). This difference was not statistically significant. However, 13 breast cancer patients (22%) but only 2 controls (3%) had K19 mRNA levels above the established reference range. This difference was statistically significant ($p = 0.001$). The actual level of K19 mRNA was statistically significant as well; the mean level in breast cancer patients was 1.97 ± 0.33 ng SKBR3 cell equivalents, while the level in normal controls was 1.00 ± 0.11 ng SKBR3 cell equivalents ($p = 0.0085$). These results suggest that on average, breast cancer patients have more circulating epithelial cells than normal individuals.

We also compared the difference in epithelial cell enrichment methods and found there was no significant difference between positive and negative selection methods in the level of K19 mRNA detected. However, there was a trend toward higher mean K19 mRNA in the breast cancer cases and lower K19 mRNA in the normal controls with the negative selection method that paralleled our previous findings with flow cytometry.

Task 3: To apply the test to treated breast cancer patients to determine if the test can predict disease progression

So far we have collected multiple specimens on 22 patients (17 patients have had two collections, 2 patients have had 3 collections, and 3 patients have had 4 collections). Of these, we have results K19 qRT-PCR results for 17 patients with two serial collections, one with three, and 2 patients with four. Out of the 20 patients with serial data available, 6 have had persistently detectable K19 mRNA in their peripheral blood. The current average follow-up time is 7.6 months. Because we have limited data with short follow-up times, it is not yet possible to determine the significance of these findings.

Key Research Accomplishments

1. Selection of a breast cancer-specific RNA (K19) and optimization of qRT-PCR reaction
2. Selection of an internal reference RNA (β2m) and optimization of qRT-PCR reaction
3. Optimization of protocol to isolate RNA from peripheral blood
4. Establishment of working relationship with oncology clinics in Seattle from which to get patient specimens
5. Increased sensitivity of the qRT-PCR assay using positive and negative selection for epithelial cells.
6. Solved formidable technical problems with the instrument used to perform our assays
7. Determined preliminary performance characteristics of the K19 qRT-PCR assay on clinical specimens

Reportable Outcomes

Abstracts

Palomares, M. R., Gralow, J. R., Koehler, K. M., Sabath, D. E. (2000) The Detection of Circulating Breast Cancer Cells Using Quantitative Cytokeratin-19 mRNA Real-Time PCR. Submitted to the Year 2000 Smith-Kline National Medical Oncology Fellows' Forum.

Palomares, M. R., Gralow, J. R., Koehler, K. M., and Sabath, D. E. (2000) Detection of Circulating Breast Cancer Cells Using Quantitative Cytokeratin-19 mRNA Real-Time PCR. Submitted to the MD Anderson Cancer Center Medical Oncology of Breast Cancer Fellows Program.

Palomares, M. R., Kussick, S. J., Koehler, K. M., Sabath, D. E. (2002) Comparison of quantitative real-time RT-PCR and flow cytometry for the detection of circulating breast cancer cells using positive versus negative epithelial enrichment methods. Int. J. Biomarkers 17(S2): S45.

Palomares, M. R., Richardson-Lander, A., Koehler, K. M., Gralow, J. R., Sabath, D. E. (2002) Quantitative real-time RT-PCR for the detection of circulating breast cancer cells: correlation with stage and treatment. Breast Cancer Research and Treatment 217.

Presentations

Palomares, M. R., Gralow, J. R., Koehler, K. M., Sabath, D. E. "The Detection of Circulating Breast Cancer Cells Using Quantitative Cytokeratin-19 mRNA Real-Time PCR," Year 2000 Smith-Kline National Medical Oncology Fellows' Forum, poster presentation on March 3, 2000.

Palomares, M. R., Gralow, J. R., Koehler, K. M., Sabath, D. E. "The Detection of Circulating Breast Cancer Cells Using Quantitative Cytokeratin-19 mRNA Real-Time PCR," MD Anderson Cancer Center Medical Oncology of Breast Cancer Fellows Program, platform presentation on July 29, 2000.

Palomares, M. R. "Minimally Invasive Biomarkers for Breast Cancer Prevention and Early Detection," Fred Hutchinson Cancer Research Center Clinical Research Division Seminar, Aug 14, 2000.

Palomares, M. R. "Minimally Invasive Biomarkers for Breast Cancer Prevention and Early Detection," Harbor-UCLA Oncology Grand Rounds, Sept 15, 2000.

Palomares, M. R. "Minimally Invasive Biomarkers for Breast Cancer Prevention and Early Detection," City of Hope Center for Cancer Genetics Technology Transfer Research Seminar, Sept 19, 2000.

Palomares, M. R. "Tumor marker development: cytokeratin real-time PCR," Department of Laboratory Medicine Research Conference, Jan. 24, 2001.

Palomares, M. R., Kussick, S. J., Koehler, K. M., Sabath, D. E. (2002) Comparison of quantitative real-time RT-PCR and flow cytometry for the detection of circulating breast cancer cells using positive versus negative epithelial enrichment methods. NCI-EORTC Cancer Diagnostics Meeting June 2002.

Palomares, M. R., Richardson-Lander, A., Koehler, K. M., Gralow, J. R., Sabath, D. E. (2002) Quantitative real-time RT-PCR for the detection of circulating breast cancer cells: correlation with stage and treatment.

Training supported by this award

Dr. Melanie Palomares, a Senior Fellow in Medical Oncology and Medical Genetics, conducted this research project in fulfillment of the research requirements of both fellowships, and partial fulfillment of her requirements for board eligibility in Hematology-Oncology.

Joyce Addo, an undergraduate at Delaware State University, worked in the laboratory during the summer of 2001 under the direct supervision of Dr. Palomares. She presented a poster of her work to our research group, to a larger audience at the University of Washington School of

Medicine as part of the UW STAR/BRIDGES Summer Research Program, and then presented her poster upon returning to college in Delaware.

Conclusions

The work accomplished so far has been to validate an assay to quantitate K19 RNA in the blood of breast cancer patients, which should reflect the number of circulating breast cancer cells. We have demonstrated that we are able to detect 1 breast cancer cell in 10^7 white blood cells. By performing epithelial enrichment by depletion of white blood cells, we achieve maximum sensitivity of this assay. We are currently acquiring patient specimens to determine whether this assay has clinical utility for both determining a patient's prognosis at initial presentation and predicting the likelihood of progressive disease on follow-up. If our efforts are successful, this assay may come to be used in the routine care of cancer patients. We hope that by detecting cancer cells in the blood, patients may be treated earlier for recurrent/metastatic disease and that this will translate into improved patient survival.

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